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DOI:

[10.1016/j.nano.2019.04.013](https://doi.org/10.1016/j.nano.2019.04.013)

*Document Version*

Peer reviewed version

[Link to publication record in King's Research Portal](#)

*Citation for published version (APA):*

Harper, R. A., Petersen, L., Saleh, M., Proctor, G. B., Carpenter, G. H., Gambogi, R., Hider, R., & Jones, S. A. (2019). Targeting macrophages and their recruitment in the oral cavity using swellable (+) alpha tocopheryl phosphate nanostructures. *Nanomedicine : nanotechnology, biology, and medicine*, 21, [102010]. <https://doi.org/10.1016/j.nano.2019.04.013>

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# Targeting macrophages and their recruitment in the oral cavity using swellable (+) alpha tocopheryl phosphate nanostructures

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**Word count for Abstract: 147**

**Word count for manuscript: 5000**

**Number of References: 36**

**Number of figures: 5**

**Number of tables: 0**

**Number of Supplementary online-only files, if any: 1**

**Funding information-** The study was financed by an Engineering and Physical Sciences Research Council (EPSRC) CASE award with Johnson and Johnson. EPSRC had no role in study design, data collection and interpretation, or the decision to submit the work for publication. Johnson and Johnson had a role in some experimental design and data interpretation.

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## Abstract

The phosphorylation of (+) alpha tocopherol produces adhesive nanostructures that interact with oral biofilms to restrict their growth. The aim of this work was to understand if these adhesive (+) alpha tocopheryl phosphate ( $\alpha$ -TP) nanostructures could also control macrophage responses to the presence of oral bacteria. The (+)  $\alpha$ -TP planar bilayer fragments ( $175 \text{ nm} \pm 21 \text{ nm}$ ) formed in a Trizma® / ethanol vehicle swelled when exposed to the cell lines (maximum stabilised size =  $29 \text{ }\mu\text{m}$ ). The swelled (+)  $\alpha$ -TP aggregates showed selective toxicity towards THP-1 macrophages ( $\text{LD}_{50} = 304 \text{ }\mu\text{M}$ ) compared to human gingival fibroblasts (HGF-1 cells;  $\text{LD}_{50} > 5 \text{ mM}$ ), and they inhibited heat killed bacteria stimulated MCP-1 production in both macrophages (control  $57.3 \pm 18.1 \text{ pg/ mL}$  vs (+)  $\alpha$ -TP  $6.5 \pm 3.2 \text{ pg/ mL}$ ) and HGF-1 cells (control  $673.5 \pm 133 \text{ pg/ mL}$  vs (+)  $\alpha$ -TP -  $463.9 \pm 68.9 \text{ pg/ mL}$ ).

Key words: Alpha tocopheryl phosphate, aggregate swelling, human gingival fibroblasts, human peripheral blood monocytes, selective toxicity, monocyte chemoattractant 1.

## Background

Macrophages perform a number of important regulatory functions in the human body<sup>1</sup>, but in several pathologies including, malignant tumours<sup>2</sup>, inflammatory disease<sup>3</sup>, metabolic disease<sup>4</sup>, infections<sup>5</sup>, and periodontitis<sup>6</sup>, their dysfunction is thought to contribute to disease progression<sup>7</sup>. Anti-cytokine therapies can act to counteract macrophage dysfunction, but their ‘off-target’ side-effects render the currently available agents inappropriate for this indication<sup>8</sup>. Macrophages are highly mobile and thus they are difficult to specifically target through traditional drug delivery approaches<sup>9</sup>. However, their ability to actively recognise and phagocytose foreign material provides a potential route to specifically deliver agents into macrophages using bespoke drug carrier systems.

One class of lipids that have the potential to be formed into materials that could target macrophages is the tocopherol lipids<sup>10</sup>. In recent work a novel tocopherol analogue, (+) alpha tocopheryl phosphate ( $\alpha$ -TP) was synthesised and was shown to form oral bio-retentive nanomaterials that disrupt biofilm growth<sup>11</sup>. In the mouth the ionic phosphate moiety of  $\alpha$ -TP interacts with simple electrolytes and this gives it the potential to swell and change shape<sup>12</sup>, which could facilitate macrophage phagocytosis and release of the active from the nanomaterial structure. However, the ability of  $\alpha$ -TP nanomaterials to selectively target macrophage responses in the mouth has yet to be tested.

In the mouth, macrophages and their cytokine products play an important role, along with enzymes, in both periodontal soft tissue and jawbone destruction<sup>13</sup>. As a consequence, macrophages and the cytokines that stimulate macrophage recruitment (*e.g.*, MCP-1<sup>14</sup>), have become targets in the search for new agents to improve oral health<sup>15</sup>. Therefore, the aim of this work was to understand if (+)  $\alpha$ -TP nanomaterials could be used to target macrophage dysfunction in the mouth. It was predicted that the surface charge and size of the aggregates

would influence their uptake into oral tissue, therefore three types of tocopherol aggregates were used in this study: (+)  $\alpha$ -T, which was predicted to display a neutral surface, and two structural isomers of  $\alpha$ -TP, the (+) isomer and ( $\pm$ ) isomer of  $\alpha$ -TP, both of which were predicted to be negatively charged in the mouth.

## Materials

(+)  $\alpha$ -T (type (VI), ~ 40%), phosphorous oxychloride ( $\text{POCl}_3$ ) ( $\geq 99\%$ ), tetrahydrofuran (THF) (anhydrous) ( $\geq 99.9\%$ ), trifluoroacetic acid (TFA) ( $\geq 99\%$ ), ( $\pm$ )  $\alpha$ -T ( $\geq 96\%$ ), Trizma® hydrochloride (Tris(hydroxymethyl)aminomethane hydrochloride) ( $\geq 99\%$ ), ( $\pm$ )  $\alpha$ -TP ( $\geq 97\%$ ), chlorhexidine digluconate (CHX) (20% w/v aqueous solution), cetylpyridinium chloride monohydrate (CPC) (99.0-102%), Hanks Balanced Salt Solution (HBSS) (Cat No H6648), 2-mercaptoethanol (99%), Dulbecco's Phosphate Buffered Saline (PBS) (cat No D8537), trypan blue (0.4%), Phorbol 12-myristate 13-acetate (PMA) ( $\geq 99\%$ , film), trypsin-EDTA (0.25%) and TRI-Reagent® (Ambion) were purchased from sigma Aldrich, UK. Hexane by fractions, absolute ethanol isopropanol, dimethyl sulfoxide (DMSO), disodium hydrogen phosphate ( $\geq 99\%$ ), monosodium dihydrogen phosphate ( $\geq 99\%$ ), hydrochloric acid, sodium hydroxide, heat-inactivated Fetal Bovine Serum (FBS), penicillin G-streptomycin, MagMax™-96 for Microarrays Kit (Ambion, AM1839), High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) were purchased from Fisher Scientific Ltd, UK. De-ionised water was used from laboratory supply. Dulbecco's modified Eagle's medium (DMEM) (ATCC 30-2002) cell culture medium and HGF-1 (ATCC CRL-2014) cells were purchased from the American Type Culture Collection (ATCC) (USA). Roswell Park Memorial Institute (RPMI) -1640 Medium (ATCC 30-2001) and the immortalised human peripheral blood monocyte cell line THP-1 (ATCC TIB-202) was sourced from LGC standards (UK). A colorimetric one step cell viability assay using a novel

tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS) was purchased from Promega, USA. This has been previously used to assess toxicity of zinc oxide nanoparticles <sup>16</sup>. Commercial enzyme-linked immunosorbent assays (ELISA's) including human MCP-1/ CCL2, human IL-8 and human IL-6 max deluxe sets were purchased from Biolegend, USA. Disposable cuvettes (Macro, PMMA), tissue culture flasks (25 and 75 cm<sup>2</sup> with ventilated caps) and 96-well plates were supplied by VWR (Germany).

## Methods

### (+) $\alpha$ -TP Synthesis

(+)  $\alpha$ -TP synthesis used phosphorus oxychloride with triethylamine in anhydrous THF for 3 hours at room temperature as previously described <sup>11</sup>. The pure product was obtained after C18 column chromatography. (+)  $\alpha$ -TP was considered sterile due to the presence of 70% isopropanol in the purification step.

### Characterisation of (+) $\alpha$ -T, (+) $\alpha$ -TP, ( $\pm$ ) $\alpha$ -TP aggregates in cell culture medium

The size of the aggregates were monitored in cell culture media to mimic the gum tissue environment. The volume median diameters of (+)  $\alpha$ -T, ( $\pm$ )  $\alpha$ -TP and (+)  $\alpha$ -TP were measured using a laser diffraction technique (Mastersizer X, version 2.15, Malvern Instruments, UK). The vitamin E derivatives (3 mM) dispersed initially in 20% ethanol, 80% water vehicles with 150 mM Trizma®. Samples were diluted in cell culture medium (DMEM or RPMI without phenol red, FBS, or pen strep) to reach an obscuration *ca* 20%. The volume median diameters were recorded every 3 minutes for 15 minutes (N = 3).

## **Cultivation of gingival fibroblast cells and peripheral blood monocyte cells**

Two different cell lines HGF-1 and THP-1 important in gum disease were employed in the study <sup>17,18</sup>. HGF-1 cells were cultured in DMEM, which included 10% FBS, and 1% of penicillin G-streptomycin (complete media A). The immortalised human peripheral blood monocyte cell line THP-1 was cultured in RPMI-1640 medium, which included 10% FBS, 1% of penicillin G-streptomycin and 50  $\mu$ M 2-mercaptoethanol (antioxidant) (complete media B). Both cell lines were grown at 37°C in a 5% CO<sub>2</sub>, humidified atmosphere until HGF-1 cells reached confluence or until THP-1 monocytes reached 1x10<sup>6</sup> cell/ mL. HGF-1 cells were subcultured using trypsin-EDTA (0.25%) (5 mL) and reseeded at 50% cell density (*i.e.* 1 flask of confluent cells were split into 2 flasks). THP-1 monocytes were subcultured every 2-3 days (not allowed to exceed 1 x 10<sup>6</sup> cell/ mL) and were reseeded at 2 x 10<sup>5</sup> cells/ mL. HGF-1 and THP-1 cells used for experiments were between passages 3-8.

## **Preparation of heat killed bacteria**

Oral heat killed bacteria was used to model the inflammatory environment found *in vivo* <sup>19, 20</sup>. Human saliva (1 donor) was collected, formed into bacterial pellets, re-suspended in cell culture media (1 mL) (DMEM or RPMI-1640) and heat killed using a heating block (Grant, QBA1 series), UK) at 80 °C for 10 minutes. The heat killed bacteria was diluted to 0.18 OD<sub>620</sub> with cell culture medium under sterile conditions followed by a 1/100 dilution of the solution in cell culture medium to form the inflammatory stimuli.

## **Determination of HGF-1 and THP-1 cell line viability**

The effects of (+)/ (±)  $\alpha$ -TP, (+)  $\alpha$ -T and heat killed human saliva bacteria, individually and in combination, were tested on HGF-1 and THP-1 cells. CHX and CPC were also tested on THP-1 macrophages as controls. To perform these assessments HGF-1 cells (passages 3-8)

were seeded ( $1 \times 10^4$  cells/ well)<sup>21</sup> in 96 well microplates (100  $\mu$ L/ well) and were incubated for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere to allow for cell adhesion. The culture medium was then aspirated, and the cells were treated for 4 h with (+)  $\alpha$ -TP (0.05, 0.5, 5, 50, 500, or 5000  $\mu$ M), (+)  $\alpha$ -T (500 or 5000  $\mu$ M) or ( $\pm$ )  $\alpha$ -TP (500  $\mu$ M) (100  $\mu$ L/ well) after which the test samples were aspirated and the cells washed with HBSS (200  $\mu$ L/ well). Either complete media or heat killed human bacteria from human saliva in complete media (1/100 dilution) was applied (100  $\mu$ L/ well) and the samples were incubated for an additional 15 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The THP-1 monocytes were seeded ( $1 \times 10^4$  cells/ well) with PMA (5 ng/ mL) to allow monocyte differentiation and subsequent cell adhesion in 96 well microplates and were incubated for 48 h at 37°C in a 5% CO<sub>2</sub> atmosphere<sup>22</sup>. The culture medium was aspirated, cells were washed with HBSS, and cultured in serum free media for 3 h. The cells were then treated for 2 h (the time was reduced from 4 h to reduce the time the cells were starved from FBS)<sup>23</sup> with (+)  $\alpha$ -TP (0.05, 0.5, 5, 50, 500, or 5000  $\mu$ M), ( $\pm$ )  $\alpha$ -TP (500  $\mu$ M), (+)  $\alpha$ -T (500 or 5000  $\mu$ M), CHX (50, 100, 150, 500, or 5000  $\mu$ M) or CPC (0.05, 0.5, 5, 50, or 500  $\mu$ M) (100  $\mu$ L/ well) after which the test samples were aspirated, and the cells washed with HBSS (200  $\mu$ L/ well). Either complete media or heat killed human bacteria from human saliva in complete media (1/100 dilution) were applied (100  $\mu$ L/ well) and incubated for an additional 15 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The 15 h supernatants were removed and used for ELISA assays and fresh complete media (100  $\mu$ L) was added to the wells containing the colorimetric MTS tetrazolium compound (20  $\mu$ L). Plates were then incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 4 h after which time absorbance's at 490 nm (iEMS Incubator/Shaker, Thermo Scientific, UK) were measured with reference subtractions at 650 nm. Untreated control cells were assigned a value of 100% viability (negative control), cell treated with 1% triton X (dispersed in cell culture media) were assigned a value of 0% viability (positive control). All the other conditions were compared to the controls using equation 1 where ABS is the corrected



absorbance's. Results are expressed as means  $\pm$  standard deviations of triplicate assays from three different experiments. Lethal dose 50% (LD<sub>50</sub>) values were calculated using the dose response model in Origin 2016.

$$\text{Cell viability (\%)} = \frac{ABS_{\text{sample}} - ABS_{\text{positive control}}}{ABS_{\text{negative control}} - ABS_{\text{positive control}}} \times 100 \quad \text{Equation 1}$$

### **Determination of cytokine secretion**

MCP-1, IL-6 and IL-8 expression were measured in the selected cell lines in response to heat killed bacteria and this was repeated in the presence of the tocopherols in order to assess their anti-cytokine effects <sup>24</sup>. Enzyme Linked Immunoassay (ELISA) kits were used to quantify the protein concentrations of MCP-1, IL-8 and IL-6 produced in the microplate cell supernatants according to the manufacturer's protocols. Preliminary experiments showed that there was no detectable MCP-1 in the heat killed bacteria applied to the cells and hence the detected MCP-1 was solely generated from the HGF-1 cells. The test solutions at a concentration of 500  $\mu$ M were not toxic against HGF-1 cells and hence this concentration was selected to assess their effect on MCP-1 release.

### **mRNA expression assay**

mRNA transcript expression assays were performed using q-PCR on HGF-1 cells to understand how MCP-1, IL-8 and IL-6 expression was being regulated in the presence and absence of the three different tocopherol aggregates. The HGF-1 cells were cultured and treated using the same method as the cell viability assay with the exception that after the 15 h incubation with the inflammatory stimuli supernatants were removed, the cells washed with PBS (100  $\mu$ L/ well) and then harvested with TRI-Reagent® (100  $\mu$ L/ well). Cell treatments groups were in quintuplet and were combined in micro centrifuge tubes (500  $\mu$ L, 50,000 cells) (N = 3). The total RNA was extracted using MagMax™-96 for Microarrays Kit. RNA was

quantified using the NanoDrop (Thermo Scientific, UK). RNA integrity was analysed using the Bioanalyzer (Agilent, UK). A 50 ng aliquot of RNA per sample was reverse transcribed to cDNA using High-Capacity RNA-to-cDNA™ Kit. Expressions of MCP-1, IL-6 and IL-8 were analysed using probes from the Universal Probe Library (UPL, Roche). Actin beta (ACTB), Selenocysteine lyase (SCLY) and tRNA-yW synthesizing protein 1 homolog (TYW1) were used as reference genes (for primer sequence and probe selection, see supplementary material, Tables S1 and S2). Assays were designed following instructions from the Universal Probe Library Assay Design Centre <sup>25</sup>. Quantitative PCR (qPCR) was performed using TaqMan Universal PCR Master Mix, following manufacturer's protocol. Each 10 µL reaction contained 0.2 µM forward primer and reverse primer and 0.1 µM UPL probe. cDNA was diluted 10-fold, and 4 µL of diluted cDNA was used per reaction. qPCR was performed on Applied Biosystems 7900HT Real-Time PCR System under the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data was collected at the end of each cycle.

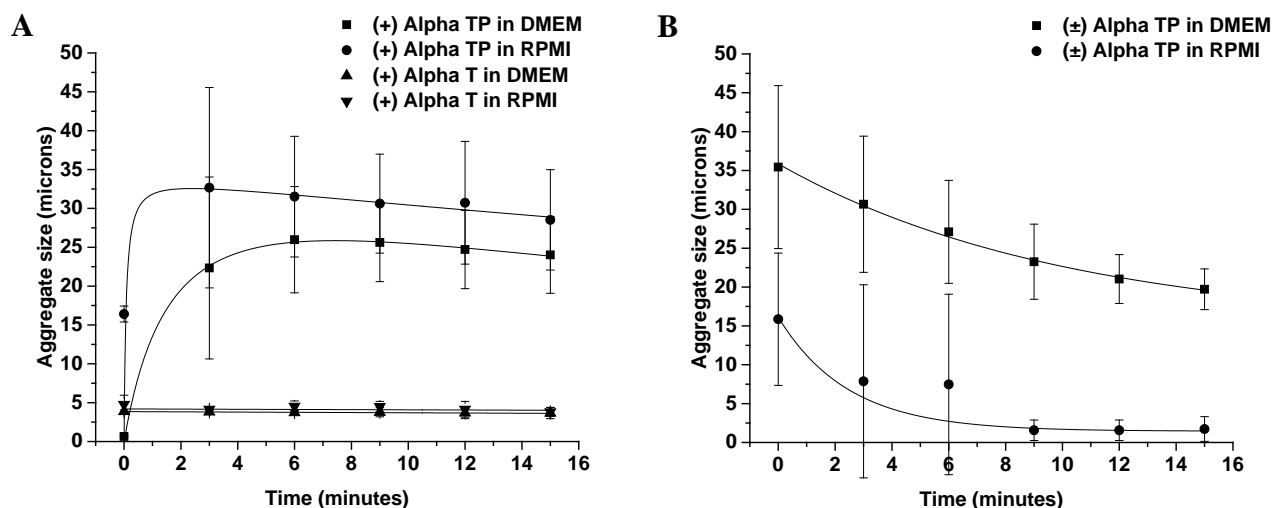
## **Data analysis**

All data was expressed as their mean  $\pm$  standard deviation (SD) at N = 3. The N numbers were independent experiments, each N number had three data points (three wells). Statistical analysis of the data was performed using Levine's homogeneity test before statistical significance between the sample groups was assessed by one way analysis of variance (ANOVA) tests with post-hoc Tukey analysis in Origin 2016 (Silverdale scientific ltd, UK). Statistically significant differences were identified when  $p \leq 0.05$ .

## Results

### Aggregate characterisation in cell culture media

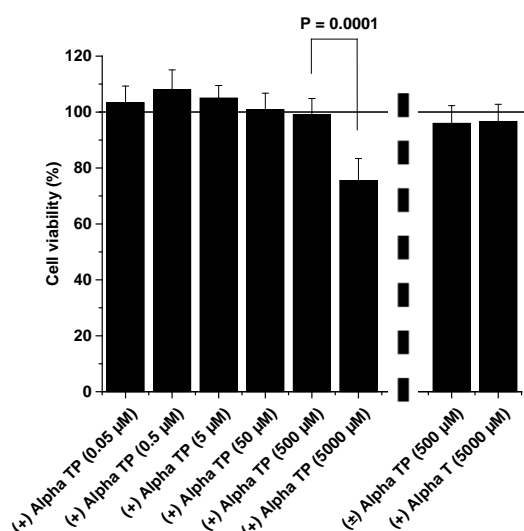
Dynamic light scattering size analysis, zeta potential, atomic force microscopy (AFM) imaging and fluorescence spectroscopy measurements of the aggregates in 20% ethanol, 80% water, 150 mM Trizma®, pH 7.4 vehicles is reported in the supplementary material (See supplementary material, Figures S1-S4)<sup>11, 12</sup>. The characterisation data showed (+)  $\alpha$ -T produced spherical liposomes ( $563 \pm 1$  nm,  $-10.5 \pm 0.2$  mV), (+)  $\alpha$ -TP produced planar bilayer fragments ( $175$  nm  $\pm$   $21$  nm,  $-14.9 \pm 3.5$  mV) and ( $\pm$ )  $\alpha$ -TP produced spherical liposomes ( $104$  nm  $\pm$   $1.3$  nm,  $-38.7 \pm 7.0$  mV) in an 20% ethanol 80% Trizma® buffer vehicle. The (+)  $\alpha$ -TP nanomaterials size was shown to increase into the micron range when aliquoted into both the DMEM and RPMI cell culture media, [with no statistically significant difference in the aggregate sizes between the two media](#) ( $P > 0.05$ ) ( $24.0 \pm 4.9$   $\mu$ m and  $28.52 \pm 6.46$   $\mu$ m at 15 min). The (+)  $\alpha$ -T aggregates swelled in both DMEM and RPMI over a period of 15 min to sizes of  $3.7 \pm 0.7$   $\mu$ m and  $3.8 \pm 0.6$   $\mu$ m respectively, but unlike (+)  $\alpha$ -TP this size did not change beyond the 15 min time point. The ( $\pm$ )  $\alpha$ -TP isomer immediately swelled to a size of  $35 \pm 10$   $\mu$ m in DMEM and  $16 \pm 9$   $\mu$ m in RPMI and then the size started to reduce upon further incubation (Figure 1 B).[It was not possible to gain clear images of the swelled aggregates in the cell culture media.](#)



**Figure 1:** The size changes of stereo-pure alpha tocopheryl phosphate ((+) Alpha TP) (3 mM) (A), (+) alpha tocopherol ((+) Alpha T) (3 mM) (A) and racemic alpha tocopheryl phosphate ((±) Alpha TP) (B) in either Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 medium to show the introduction of the phosphate group facilitates the swelling.. Data represented mean  $\pm$  standard deviation,  $n = 3$ .

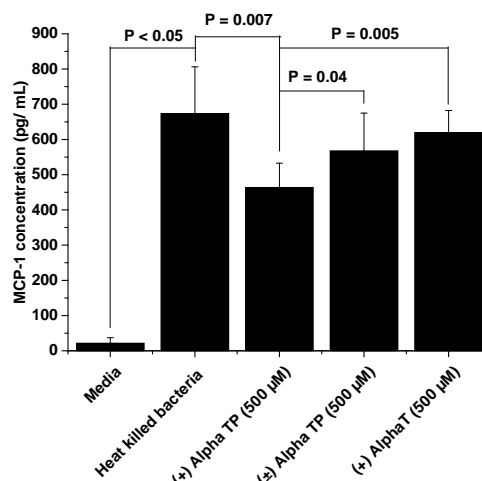
### HGF-1 cellular response to the tocopherol aggregates

(+)  $\alpha$ -TP was well tolerated by HGF-1 cells until a concentration of 5 mM (0.25% w/v) at which point the cell viability significantly dropped to  $75.5 \pm 7.9\%$  (Figure 2). Both the (±) and the (+)  $\alpha$ -TP isomer (500  $\mu$ M) were found to have the same effects on cell viability ( $P > 0.05$ ), which suggested the stereochemistry was not a factor in the cell toxicity. At 5 mM (+)  $\alpha$ -T did not reduce the cell viability ( $96.7 \pm 6.1\%$ ), which showed that the addition of the phosphate group significantly increased the agents' cell toxicity.



**Figure 2:** Human gingival fibroblast cell (HGF-1) viability upon tocopheryl application showing good tolerability to the test compounds. Cells treated with (+) alpha tocopheryl phosphate (alpha TP), (±) alpha TP or alpha tocopheryl (alpha T) diluted in Dulbecco's modified eagle's cell culture medium (without FBS) at 37 °C for 4 hours. Data represented mean ± standard deviation,  $n = 3$ .

When treated with heat killed bacteria from human saliva, HFG-1 cells demonstrated a significant increase in secretion of MCP-1 ( $673.5 \pm 133$  pg/ mL, Figure 3A) as compared to the cells only treated with media ( $21.6 \pm 15.6$  pg/ mL). (+)  $\alpha$ -T and (±)  $\alpha$ -TP (500  $\mu$ M) did not significantly inhibit MCP-1 production ( $\alpha$ -T -  $619 \pm 63$  pg/ mL,  $P > 0.05$ , (±)  $\alpha$ -TP -  $568 \pm 107$  pg/ mL,  $P > 0.05$ ). However, (+)  $\alpha$ -TP (500 $\mu$ M) did significantly reduce MCP-1 production ( $463.9 \pm 68.9$  pg/ mL,  $P = 0.007$ ). IL-8 ( $8601 \pm 600$  pg/ mL) and IL-6 ( $18310 \pm 896$  pg/ mL) were also produced in response the heat treated bacteria, but none of the tocopherol test agents were found to have an inhibitory effect on their production (See supplementary material, Figure S5).



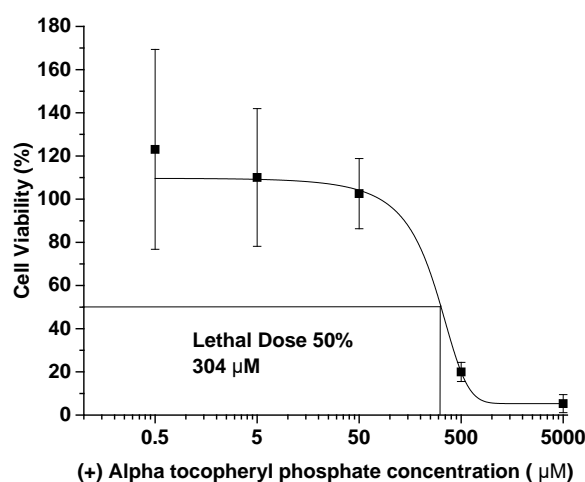
**Figure 3:** Tocopherol inhibition of monocyte chemoattractant protein-1 (MCP-1) secreted from human gingival fibroblast cells *showing (+) alpha TP was the only sample that produced a statistically significant reduction from the positive control.* Cells pre-incubated with (+) alpha tocopheryl phosphate (alpha TP), (±) alpha TP or (+) alpha tocopherol (alpha T) samples for 4 h followed by 15 hours exposure to inflammatory stimuli. Data represented mean  $\pm$  standard deviation,  $n = 3$ . *N.B. No change in cell morphology was noted by phase contrast.*

The pre-treatment of the HGF-1 cells with (+)  $\alpha$ -TP before inflammatory stimulation did not significantly reduce the mRNA transcription of MCP-1, IL-6 or IL-8 compared to the heat killed bacteria treated cells ( $P > 0.05$ , see supplementary material, Figure S6).

### THP-1 macrophage cellular response to the aggregates

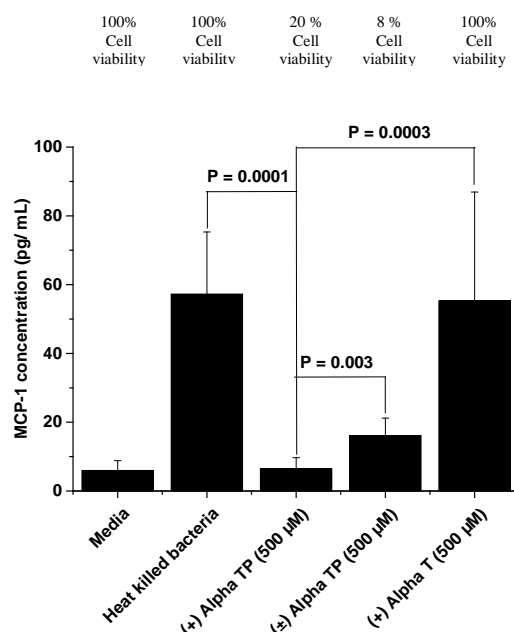
(+)  $\alpha$ -TP was less well tolerated by the differentiated THP-1 monocytes (macrophages) compared to the HGF-1 cells (Figure 4). Macrophages treated with 500  $\mu$ M of the (±)  $\alpha$ -TP and (+)  $\alpha$ -TP isomers were found only retain  $7.7 \pm 4.6$  % (data not in figure) and  $20.0 \pm 4.4$  % viability (Figure 4), respectively. The (+)  $\alpha$ -TP LD<sub>50</sub> against the macrophages was calculated to be 304  $\mu$ M. As seen with the HGF-1 cells, (+)  $\alpha$ -T (5 mM) was well tolerated by the THP-1 macrophages. Both CHX and CPC, found in commercially available mouthwash products,

showed a lower LD<sub>50</sub> value compared to (+)  $\alpha$ -TP (Supplementary data, Figure S7), which suggested that (+)  $\alpha$ -TP was better tolerated than CHX and CPC.



**Figure 4:** (+) *alpha tocopheryl phosphate* toxicity to THP-1 macrophages generated an LD50 value of 304  $\mu$ M. Data represented mean  $\pm$  standard deviation,  $n = 3$ .

THP-1 macrophages treated with the negative control (media) produced  $6.0 \pm 2.9$  pg/ mL of MCP-1 whilst the macrophages treated with heat killed bacteria generated  $57.3 \pm 18.1$  pg/ mL MCP-1 (Figure 5). (+)  $\alpha$ -T (500  $\mu$ M), which was not toxic to macrophages, was not found to inhibit MCP-1 generated from the THP-1 macrophages ( $55.3 \pm 31.7$  pg/ mL). Cells treated with ( $\pm$ ) and (+)  $\alpha$ -TP (500  $\mu$ M), which were toxic to macrophages, resulted in very low levels of MCP-1 secretion ( $16.1 \pm 5.1$  pg/ mL for ( $\pm$ ) isomer and  $6.5 \pm 3.2$  pg/ mL for (+) isomer,  $p = 0.003$ ) ((+) isomer,  $p > 0.05$  with media control). Non-toxic concentrations of ( $\pm$ ) or (+)  $\alpha$ -TP did not inhibit MCP-1 production from the macrophage cells.



**Figure 5:** Tocopherol inhibition of monocyte chemoattractant protein (MCP-1) secretion from THP-1 macrophages related to cell viability showing that cell death inhibited MCP-1 release. Cells were pre-incubated with (+) alpha tocopheryl phosphate (alpha TP), (±) alpha TP or alpha tocopherol (alpha T). Data represented mean  $\pm$  standard deviation,  $n = 3$ . *N.B. No change in cell morphology was noted by phase contrast.*

## Discussion

The tocopherol nanomaterials tested in this work swelled when spiked into cell culture media. The different swelling profiles observed for the nanomaterials in the DMEM and RPMI fluids was thought to be a consequence of the media composition. The iron, which was only present in the DMEM, was identified as the most likely component to explain the differential behaviour, as it is known to interact with phosphorylated compounds<sup>26</sup>. In both media the swelling of the nanomaterials was attributed to a transition of the lipid aggregates to multi-lamella liposomes due to the change of ionic strength in the dispersion media<sup>11</sup>. An alternative explanation to the observed size changes was that the nanomaterials were aggregating. However, this was thought to be unlikely as the dispersions changed size with a high degree of reproducibility, which is not common in aggregated systems<sup>27</sup>. Unlike macrophages, non-



phagocytic cells are unlikely to internalise the micron sized carriers, hence the changes in nanomaterial characteristics in physiological fluids were thought to be beneficial for macrophage targeting. This hypothesis was supported by the cell cytotoxicity and cytokine suppression data discussed further below.

The (+)  $\alpha$ -TP reduced MCP-1 production by HGF-1 cells exposed to heat killed bacteria by approximately 32%. In the literature there does not seem to be a report that has previously shown that  $\alpha$ -TP can suppress MCP-1 release. The anti-inflammatory agent, Bindarit, which has some structural similarities to  $\alpha$ -TP has previously been shown to selectively inhibit MCP-1 through the inhibition of MCP-1 mRNA, but in this work (+)  $\alpha$ -TP was found not to inhibit mRNA transcript expression. From the lack of MCP-1 mRNA inhibition it could be deduced that (+)  $\alpha$ -TP influenced post mRNA transcription activity such as protein synthesis or cellular protein secretion inhibition. However, because the cell signalling pathways for the heat treated saliva induced cytokine release from HGF-1 cells has not been well established the (+)  $\alpha$ -TP mechanism of action was not investigated further <sup>28</sup> because this would require the use of both pharmacological inhibitors and an investigation of the phosphorylation signals of MAPK to investigate the IL-6, IL-8 and MCP-1 release and then western blot analysis to look for protein expression changes, which we considered to be outside the scope of the current paper.

It was surprising that all the tocopherols used in this work did not inhibit cytokine release from the cell lines because (+)  $\alpha$ -T has previously been shown to have anti-inflammatory effects <sup>29,30,31</sup>. In addition, (+)  $\alpha$ -T has been observed to inhibit IL-6 from HGF-1 cells in the literature <sup>32</sup>. However, in previous work LPS from *P. gingivalis* was used to induce the cytokine and in this study was heat killed bacteria from human saliva was used. It is possible that the tocopherols inhibit cytokine production on the LPS stimulation pathway and not other salivary inflammatory stimuli pathways.

The selective  $\alpha$ -TP toxicity and subsequent reduction in MCP-1 secretion from THP-1 macrophages was thought to be a function of the nanomaterial swelling. The most selective of the aggregates in terms of  $\alpha$ -TP toxicity and MCP-1 suppression was (+)  $\alpha$ -TP followed by ( $\pm$ )  $\alpha$ -TP then (+)  $\alpha$ -T, which was not toxic and did not suppress MCP-1. This rank order aligned to the sizes of the aggregates when presented to the cells. This could, at least in part, be a consequence of greater internalisation of the larger aggregates due to more material volume being phagocytised by individual macrophages<sup>33</sup>. Selective macrophage toxicity of  $\alpha$ -TP was thought to be desirable for the treatment of the chronic inflammatory phase of periodontal disease as it could control the macrophage burden and shorten the immune response<sup>34</sup>. However, it is not desirable to kill all macrophages as they perform a beneficial role of engulfing microbes and preventing the spread of systemic infection. In this aspect it was promising that even though (+)  $\alpha$ -TP showed selective toxicity to the macrophages it was still found to be less toxic (lower LD<sub>50</sub> values) than two commercially used antimicrobial agents CHX and CPC; suggesting that it was capable of regulating macrophage accumulation without complete depletion.

## Conclusion

Mild and moderate periodontitis affects the majority of the adult global population with 10.5 - 12% of the population affected by severe periodontitis making it the sixth most prevalent condition in 2010<sup>35</sup>. However, at present, sub-antimicrobial dose doxycycline is the only agent that has been approved for human use that inhibits the gingival inflammatory process, the cause of the destructive elements of this disease<sup>36</sup>. (+)  $\alpha$ -TP has previously been shown to be tooth adherent, substantive, and capable of reducing the oral microbial burden<sup>11, 12</sup>. In this study the swelling of (+)  $\alpha$ -TP nano-sized aggregates was shown to have an anti-inflammatory effect on macrophages by selective macrophage toxicity thereby reducing MCP-1 generation. This effect would then dampen excessive macrophage burden and therefore reduce gingival destruction

making this compound an attractive prospect for development as a multifunctional agent to improve oral health.

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